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14. ABSTRACT

Lysozyme directs the formation of silica or titania nanoparticles under ambient conditions and is simultaneously entrapped while in the active bactericidal form. The ability to encapsulate an active antimicrobial protein within inorganic nanoparticles provides an opportunity to create bionanocomposite materials that resist bacterial activity, for use as broad spectrum antifouling materials.

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Rapid, Room-Temperature Synthesis of Anti-bacterial Bio-nano-composites of Lysozyme with Amorphous Silica or Titania

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The majority of natural biomineralized structures are composed of calcium carbonate or silica, which are not always well suited for biotechnological applications. While many natural silica-forming proteins have been identified,[1] only the silicatein of marine sponges has been reported to catalyze the formation of titania *in vitro*.[2] Lysozyme is a ubiquitous antibacterial enzyme capable of lysing gram-positive bacterial cells by hydrolyzing specific peptidoglycan linkages in the cell wall.[3] Recent reports indicate the involvement of lysozyme in the biomineralization of silica and calcium carbonate,[4] and heat denatured lysozyme has also been implicated in the synthesis of bismuth sulfide, but the mechanism is unclear.[5] Here a rapid, economical, room temperature method for encapsulating lysozyme within silica or titania nanoparticles, with appreciable retention of anti-microbial activity is reported for the first time.

The development of a simple, low-cost processing route to lysozyme/inorganic nanocomposites, and evaluation of their antimicrobial activity provides an opportunity to create bionanocomposite materials that resist bacterial activity for use as broad-spectrum antifouling materials. Lysozyme directs the formation of nanoparticles of silica or titania under ambient conditions, so as to simultaneously entrap the lysozyme in an active form. Amorphous titania-based materials can possess diverse optical, electronic, biomedical, and chemical properties.[6] Hence, lysozyme/titania nanocomposites could be attractive multifunctional materials for a variety of applications (e.g., for cosmetics with both antimicrobial and sunscreen properties).[7]

In the present work, lysozyme catalyzed the precipitation of silica within seconds when added to a solution of pre-hydrolyzed tetramethoxysilane (TMOS). Lysozyme also catalyzed the rapid precipitation of titania when added to a solution of either potassium hexafluorotitanate (PHF-Ti) or titanium (IV) *bis* (ammonium lactato) dihydroxide (Ti-BALDH). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) revealed that the silica consisted of interconnected nanospheres with sizes ranging from 300 to 650 nm, with an average diameter of 570 nm (Figure 1). The silica morphology was similar to that previously observed for the interaction of lysozyme with water glass.[4a] Energy dispersive spectroscopy (EDS) analysis conducted during SEM characterization indicated that the silica spheres were enriched in carbon and sulfur, as well as oxygen and silicon (Figure 2). The presence of carbon in the EDS spectrum was consistent with a precipitate consisting of an organic/inorganic composite of protein within a silica matrix. The cysteine residues in the lysozyme provided a source of sulfur. Selected area electron diffraction (SAED) analysis (inset of Figure 1c) and X-ray diffraction (XRD) analysis (not shown) indicated that the precipitated silica was amorphous. The entrapment of lysozyme within the silica-bearing precipitate was confirmed by dissolving the

silica in sodium hydroxide, to release the entrapped protein. The recovered organic component was analyzed (and compared to soluble lysozyme) using denaturing polyacrylamide gel electrophoresis (SDS-PAGE), which revealed the presence of a protein band with a molecular weight of *ca.* 14 kDa (the molecular weight of lysozyme) (not shown). The presence of a large proportion of organic matter within the precipitate was further confirmed by thermogravimetric (TG) analysis, which resulted in a 61.8% loss of dry mass upon pyrolysis in air at 700 °C. The surface area of the silicaceous material before and after heat treatment (1000 °C) was determined by multi point nitrogen adsorption Brunauer, Emmett and Teller (BET) analysis to be 6.28 and 7.42 m^2/g, respectively. The observed increase of specific surface area after pyrolysis is consistent with the presence of lysozyme, because the removal of the organic constituent would be expected to create voids and increase the surface area of the sample. SEM characterization of the pyrolyzed material indicates that the overall morphology of the silica spheres is retained despite the removal of lysozyme. Distinct to the post fired sample are dark spots (which are likely pores generated by the pyrolysis of the organic constituent of the sample) that cover the silica spheres (Supplementary Figure 1.).

The lysozyme/titania nanospheres differed markedly from the lysozyme/silica precipitates, both in their size and degree of interconnectivity. SEM analysis revealed that the lysozyme/PHF-Ti derived material was quite polydisperse, with diameters ranging from 100 nm to 1 μm. Individual lysozyme/PHF-Ti spheres were more strongly interconnected than the siliceous material, taking on a more sintered appearance (Figure 1). EDS analysis indicated that the lysozyme/PHF-Ti material was primarily composed of carbon, oxygen, titanium, potassium, phosphorus and sulfur (Figure 2). Lysozyme is the likely origin of the carbon and sulfur signal as noted above, whereas titanium, oxygen and potassium peaks are clearly associated with the

inorganic precursor. The phosphorous can be traced to the incorporation of phosphate ions from the buffer solution during precipitation. The entrapment of lysozyme within the titania precipitate was confirmed via analyses with SDS-PAGE of the organic component recovered after dissolution of the titania in sodium hydroxide (as discussed above for the silica-entrapped lysozyme). The substantial organic component of the composite was confirmed by TG analysis that resulted in a 59.3% loss in dry weight upon heating to 700 °C in air. Additional weight loss, upon heating to 700 – 900 °C, was attributed to the evaporation of potassium. Selected area electron diffraction (SAED) analysis (inset of Figure 1f) and X-ray diffraction (XRD) analysis (not shown) indicated that the precipitated material was amorphous. Subsequent heat treatment of the lysozyme/PHF-Ti composite materials at 600°C for four hours resulted in the formation of phase pure potassium titanyl phosphate (Supplementary Figure 2), a unique non-linear optical material that has found application in lasing and low loss waveguides.[8]

The morphology of titanium dioxide formed by the interaction of lysozyme and Ti-BALDH differed conspicuously from the precipitates formed from silicic acid or PHF-Ti precursors. SEM and TEM analyses of the titania precipitate revealed an open, highly-interconnected network of very fine particles. The fundamental particles entrapped within the network possess diameters of only 10-50 nm. EDS analyses of the lysozyme/TiBALDH product showed an elemental composition similar to lysozyme/PHF-Ti (Figure 2). SDS-PAGE analysis of the organic material, recovered after dissolution of the titania in sodium hydroxide, again indicated the presence of lysozyme within the composite material. The proportion of combustible components of the material was somewhat higher (76.3%, from TG analysis) than that of either previously observed lysozyme/inorganic materials. SAED analysis (inset in Figure 1i) and XRD analysis

indicated that, unlike previously generated biomimetic titania,[2] the lysozyme-catalyzed material was amorphous.

The activity of the encapsulated lysozyme was compared with that of the free enzyme to determine the effect of immobilization. The physical entrapment of lysozyme within an inorganic matrix could, in principle, inhibit the ability of lysozyme to attach to a bacterial cell wall and catalyse lysis. Two assay methods were used to determine the activity of the encapsulated lysozyme. Bacteriolytic activity was investigated with *Micrococcus lysodeikticus* cells and by hydrolysis of a synthetic membrane mimic, *p*-Nitrophenyl β-glycoside of N-acetylchitooligosaccharide $(PNP\text{-}GlcNAc)_5$).[9] Following immobilization of lysozyme in silica, 85% (± 6.6) of the free enzyme activity was retained in the silica/lysozyme nanocomposite. A portion of the remaining enzyme activity and protein (approximately 10%) was detected in the reaction supernatant and subsequent wash fractions. Following immobilization of lysozyme in titania, 50% (± 6.5) (with PHF-Ti as precursor) and 45% (±12.7) (with Ti-BALDH as a precursor) of the free enzyme activity was retained in the titania/lysozyme nanocomposite. The activity of immobilized lysozyme was comparable irrespective of the assay method investigated (Figure 3a). The thermostability of the free and immobilized lysozyme was investigated to determine whether the inorganic matrices provide an environment that protects the immobilized lysozyme from denaturation. Free lysozyme in solution was denatured by incubation at 75 °C for 1 hour (90% decrease in activity). In comparison, the silica and titania encapsulated lysozyme retained 75% and 20-45% of the native activity respectively, when incubated under the same conditions (Figure 3b).

The possibility of immobilizing an additional enzyme (for enhanced functionality) during the lysozyme-mediated precipitation reactions was also investigated. Butyrylcholinesterase was

selected as a suitable model enzyme for immobilization due to its versatility in biosensor applications.[10] Under precipitation conditions as described above, butyrylcholinesterase encapsulated during silica and titania precipitation fully retained the native enzyme activity (Figure 3c). Lysozyme-induced immobilization provided a more economical and efficient encapsulation of butyrylcholinesterase than we had previously reported using a synthetic silica-forming peptide.[11]

Lysozyme is a cationic polypeptide with a high isoelectric point (pI ≈ 10.5) and includes a large percentage of hydroxyl- and imidazole- containing amino acid residues characteristic of many biomineralization-mediating biomacromolecules.[1,12] The ability to immobilize lysozyme has been demonstrated using a variety of support matrices, all involving attachment or adsorption to a surface.[13] The limitations associated with surface-bound lysozyme (e.g., loading capacity and poor retention of activity) were overcome here by partially encapsulating the lysozyme within the porous inorganic matrices, thereby retaining the native protein structure and function. The primary physiological role of lysozyme is hydrolysis of the peptidoglycan of bacterial cell walls and the resultant cell lysis (i.e., a mechanism that involves lysozyme acting in contact with bacterial cell walls). The observation that lysozyme retains its catalytic activity when encapsulated suggests an alternate mechanism for lytic action. The section of protein responsible for precipitation of silica or titania may be independent from the protein active site. The bactericidal properties of lysozyme are known to be independent of enzymatic activity, as has been confirmed by the retention of bactericidal properties in denatured lysozyme.[3,14] The mechanism of bactericidal activity in encapsulated lysozyme, however, requires further elucidation.

In summary, lysozyme induces the rapid, room-temperature precipitation of amorphous silica and titania that, in turn, provide excellent support matrices for additional enzymes added during the reaction. Such lysozyme-mediated precipitation is a one-pot procedure that simultaneously results in lysozyme encapsulation within the silica or titania, with appreciable retention of anti-microbial activity. The inorganic matrices protect the immobilized biomolecules from physical denaturation, as occurs with free enzymes in solution, and the lysozyme offers protection from microbial degradation. This attractive biomineralization/bioencapsulation strategy provides an economical and facile route for synthesizing a wide range of functional bionanocomposites comprising biomacromolecules entrained within silica or titania nanostructures.

Experimental Section

P-Nitrophenyl-penta-N-acetyl-β-chitopentaoside [PNP- $(GlcNAc)_5$] was from Seikagaku Corp, Tokyo. Lysozyme (EC 3.2.1.17) and butyrylcholinesterase (EC 3.1.1.8) were from Sigma-Aldrich. Butyrylcholinesterase stock solutions were prepared in a cholinesterase specific buffer (0.1 N NaOH, 0.1 M KH_2PO_4, pH 8). All other chemicals were of analytical grade and obtained from Sigma-Aldrich. The synthetic peptide, R5, was obtained from New England Peptide, Inc.

For precipitation of inorganics, a stock solution of lysozyme (100 mg ml^{-1}) was prepared in deionized water and dialyzed prior to use (Slide-A-Lyzer®, Pierce Biotechnology). Tetramethyl orthosilicate (TMOS) was hydrolyzed in 1 mM hydrochloric acid (1 M final concentration). The precipitation mixture consisted of 800 µl of buffer (0.1 N NaOH, 0.1 M KH_2PO_4, pH 8), 100 µl of hydrolyzed TMOS (final concentration of 100 mM) and 100 µl of lysozyme (10 mg ml^{-1} final concentration). The mixture was agitated for 5 minutes at room temperature. The resultant particles were removed by centrifugation for 10 seconds (14,000 x g) and then washed twice with deionized water. For the formation of titania particles, the precipitation reaction was as described above with either 100 mM PHF-Ti or Ti-BALDH as the inorganic precursor. Ti-BALDH stock solutions were prepared in water, PHF-Ti was prepared in water and heated gently (50 °C) to aid in solubility.

For encapsulation of butyrylcholinesterase in the precipitates, the precipitation mixture consisted of 800 µl of buffer containing butyrylcholinesterase (50 U ml^{-1}), 100 µl of precursor (concentrations described above) and 100 µl of lysozyme (100 mg ml^{-1}). Butyrylcholinesterase enzyme activity was measured spectrophotometrically at 630 nm as described previously.[11] Entrapped lysozyme was released from silica and titania particles by dissolving the inorganic matrix in 1 M sodium hydroxide for 10 min at 37 °C and the proteins were visualized by denaturing polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE (15%) was performed using a Mini-Protean II apparatus (Amersham Pharmacia) at a constant voltage of 200 V and according to the manufacturer's instructions. Lysozyme activity assays were performed with *Micrococcus lysodeikticus* cells according to the supplier's instructions. The activity of lysozyme was also determined colorimetrically using the substrate [PNP – $(GlcNAc)_5$] as reported previously.[9b]

The morphology of the lysozyme/inorganic composite materials was characterized by scanning (Leo 1530 FEG SEM, Carl Zeiss SMT AG) and transmission (JEOL 100CX II) electron microscopy. Microchemical analyses were conducted utilizing an Oxford Inca EDS detector attached to the scanning electron microscope, using at least 3 measurements for each sample. The crystalline phases of the resulting composite materials were evaluated by x-ray diffraction (Philips PW1800, PANalytical) utilizing Cu Kα radiation at a scan rate of 0.6° $minute^{-1}$. Thermo gravimetric analyses (TGA) were performed in a Netzsch STA 449 C by heating to 1000°C at a rate of 10°C $minute^{-1}$ in a synthetic air gas stream. Multi point nitrogen adsorption BET analysis was conducted in a Quantachrome Autosorb-1c on 120°C dried samples.

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Figure 1 SEM and TEM micrographs of silica and titania nanoparticles formed by precipitation with lysozyme

a, b) SEM and **c**) TEM micrographs of silica from lysozyme and TMOS; **d, e**) SEM and **f**) TEM micrographs of titania from lysozyme and PHF-Ti; **g, h**) SEM and **i**) TEM micrograph of titania from lysozyme and Ti-BALDH. **Insets**; SAED pattern of precipitates

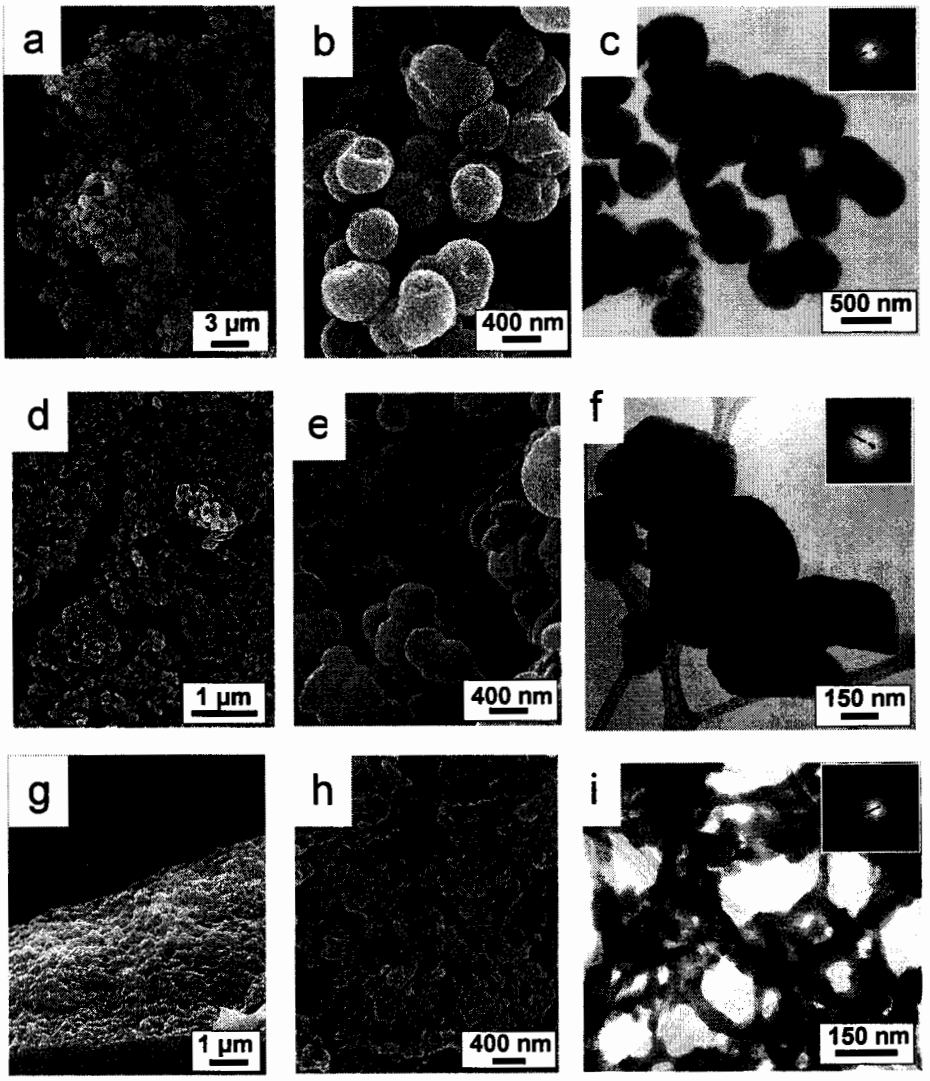


Figure 2. Energy dispersive spectroscopy of lysozyme-mediated silica and titania precipitates

(**a**) Silica precipitated with lysozyme and TMOS; (**b**) Titania precipitated with lysozyme and PHF-Ti; (**c**) Titania precipitated with lysozyme and Ti-BALDH

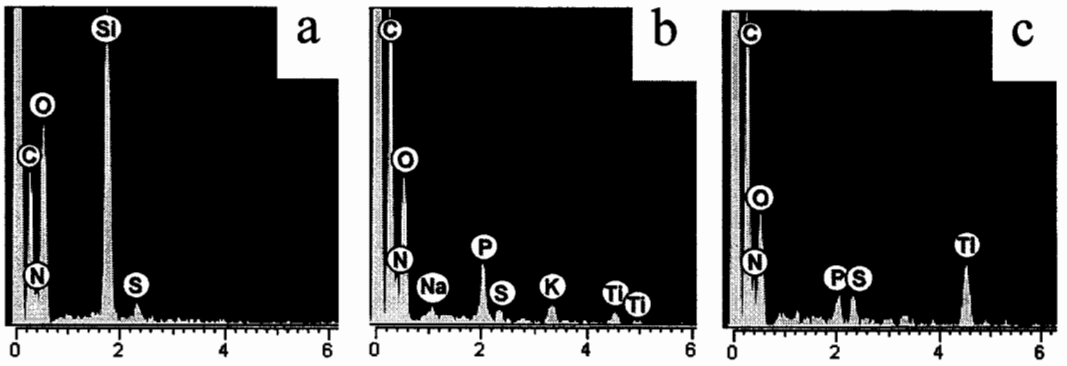
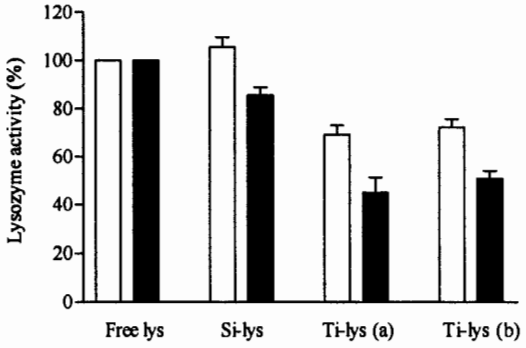
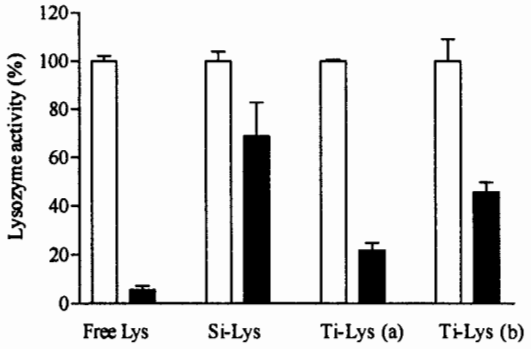


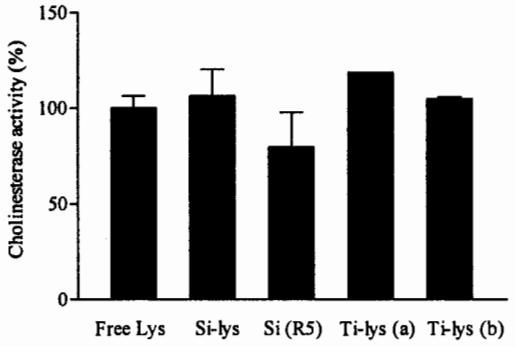
Figure 3a. Activity of free and immobilized lysozyme in silica or titania nanoparticles
Lysozyme activity determined by lytic assay with *Micrococcus lysodeikticus* cells (□) or with PNP-$(GlcNAc)_5$ (■). Values quoted are relative to the activity of native lysozyme in solution, taken as 100%



b. Thermostability of free and immobilized lysozyme in silica or titania nanoparticles
(□) Activity before heat treatment, (■) Activity following heat treatment (1 hour, 75 °C)



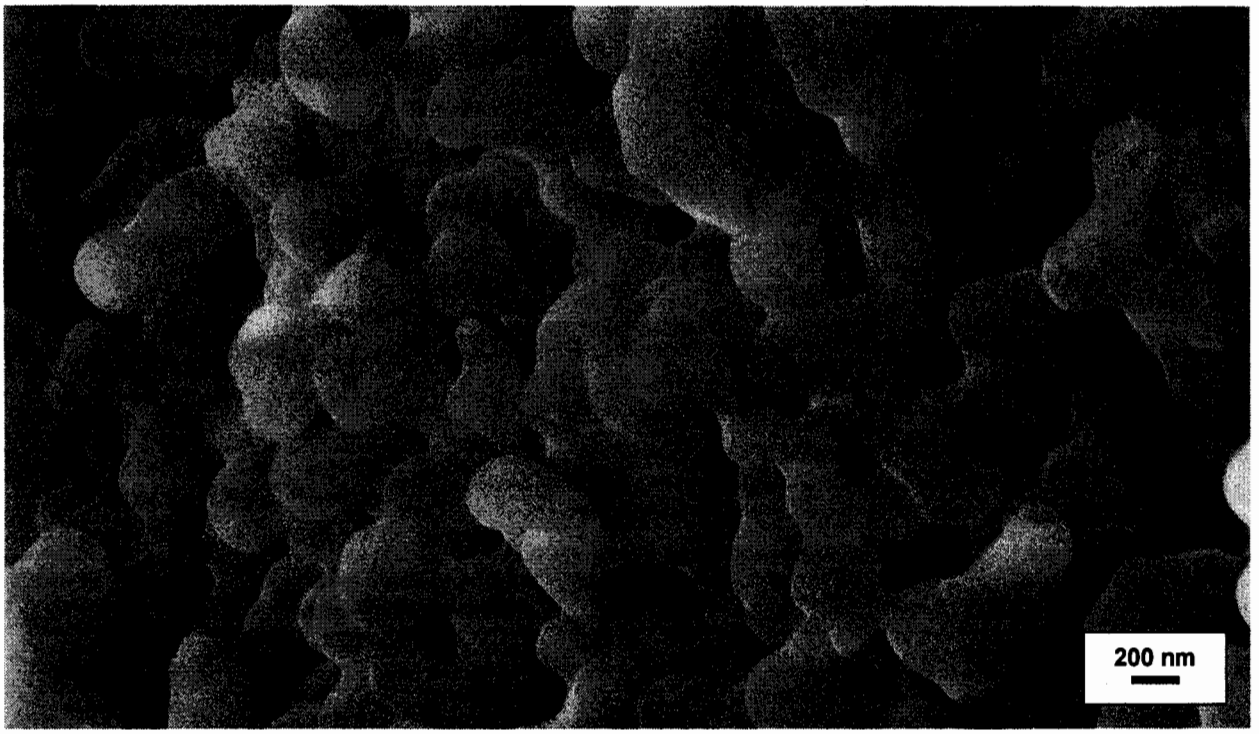
c. Activity of butyrylcholinesterase immobilized in lysozyme-catalysed silica or titania nanoparticles



Key; Free lysozyme in solution (Free Lys); Silica precipitated with lysozyme (Si-lys); Silica precipitated with R5 peptide[11] (Si-R5); titania precipitated with lysozyme and Ti-BALDH (Ti-lys (a)); titania precipitated with lysozyme and PHF-Ti (Ti-lys (b))

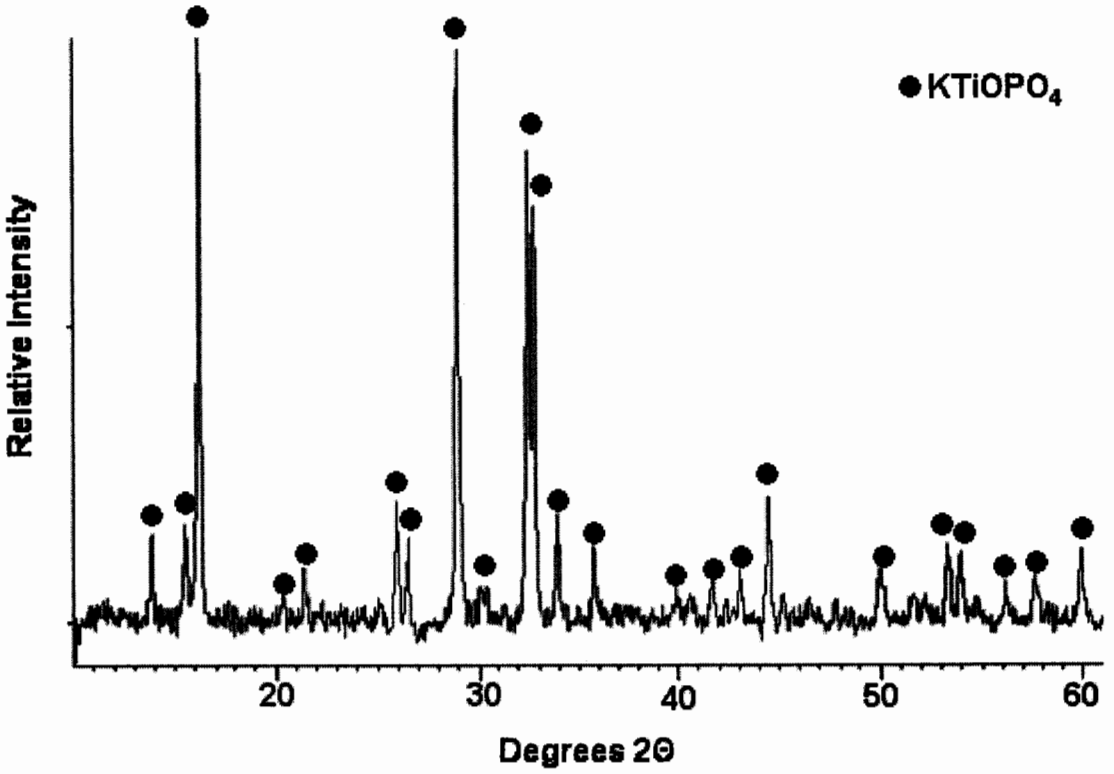
Supplementary Figure 1. SEM micrograph of lysozyme/silica nanospheres following heat treatment

SEM micrograph of silica from lysozyme and TMOS, following 1000°C heat treatment, arrow indicates an example of porosity not detected in the unfired precipitates.



Supplementary Figure 2. XRD pattern of phase pure $KTiOPO_4$

Lysozyme/Titania from PHF-Ti heat-treated at 600°C for 4 hours



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Rapid, Room-Temperature Synthesis of Anti-bacterial Bio-nano-composites of Lysozyme with Amorphous Silica or Titania

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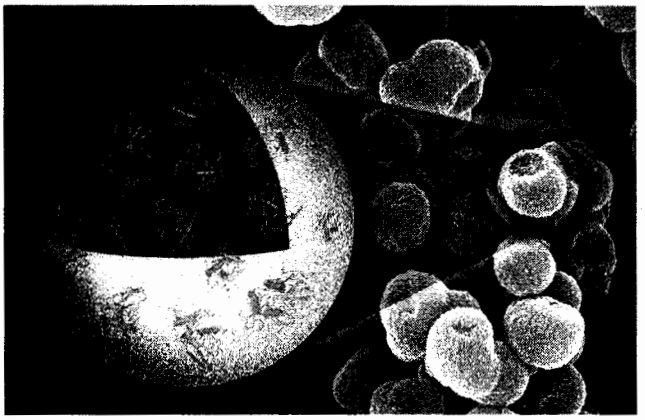
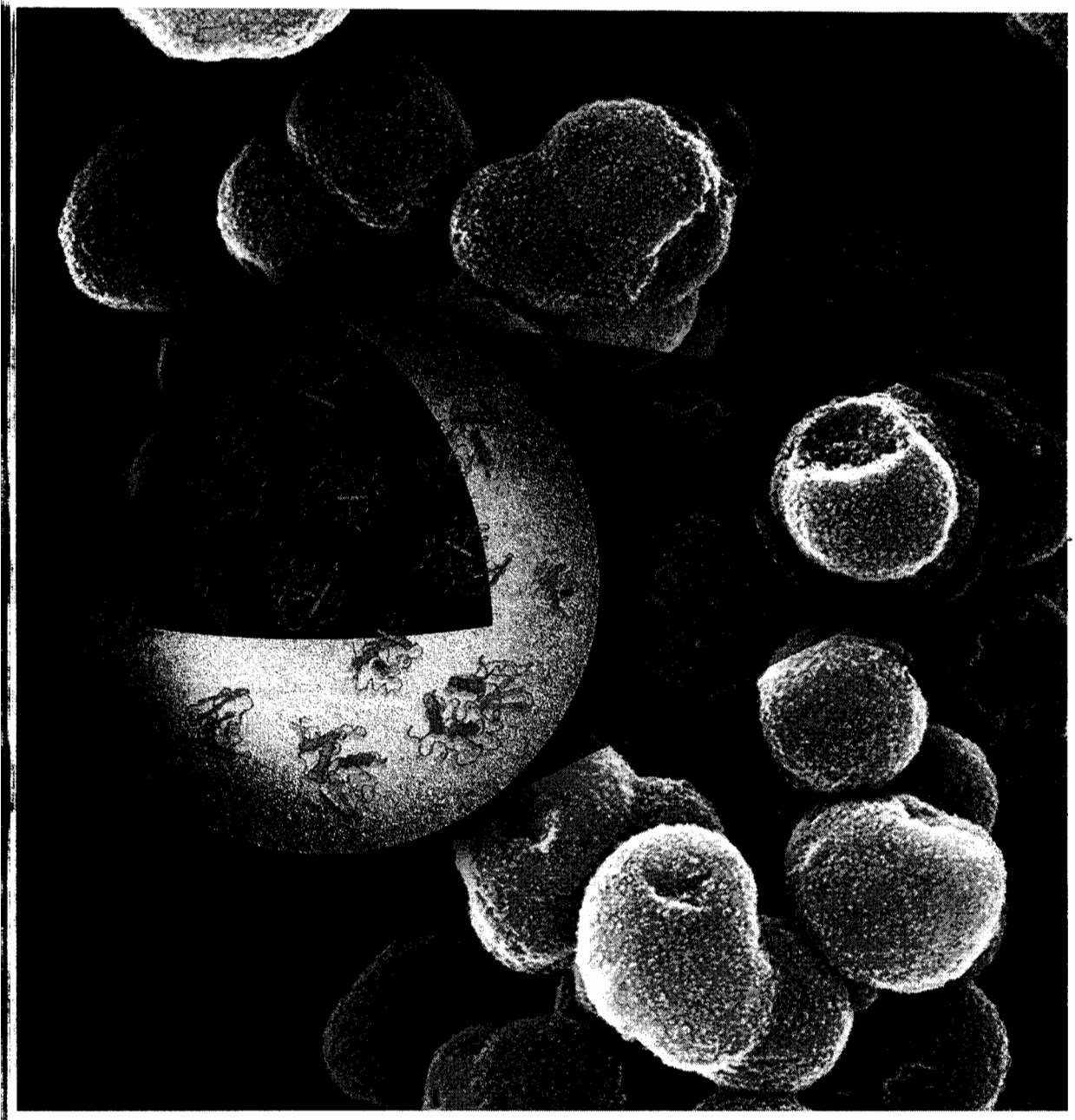


Inorganic bactericidal nanocomposites: Lysozyme directs the formation of silica or titania nanoparticles under ambient conditions, simultaneously entrapping the enzyme in its active bactericidal form. The ability to encapsulate an active antimicrobial protein within inorganic nanoparticles provides an opportunity to create bionanocomposite materials that resist bacterial activity for use as broad-spectrum antifouling materials.

Keywords:

Biomineralization, Nanomaterials, Lysozyme, Biomimetic synthesis, Enzyme Immobilization



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